

APPENDIX

DYNAMICS OF 6-METHOXYBENZOXAZOLINONE IN WINTER WHEAT Effects of Photoperiod and Temperature

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Abstract—6-Methoxybenzoxazolinone (6-MBOA), a compound derivable from some freshly growing plants, is known to stimulate reproduction in some mammals and birds. Winter wheat was studied under controlled laboratory conditions to determine the effects of photoperiod and temperature on derivable 6-MBOA content. Longer photoperiods decrease the amount of derivable 6-MBOA per gram of fresh material in 4-day-old wheat seedlings. Higher temperatures also decrease the amount of derivable 6-MBOA in 4-day-old wheat. 6-MBOA content decreases as the plant ages. Comparisons of only the first centimeter above the seed produced the same age-related result. 6-MBOA is concentrated in the meristematic region with decreasing amounts found in higher portions of the plant. Roots from 9-day-old plants contain 6-MBOA. Unsprouted wheat seeds contain negligible amounts of 6-MBOA. These results demonstrate that environmental variables have a significant effect on derivable 6-MBOA levels, but that under all the regimes studied, 6-MBOA is present in freshly sprouted wheat.

Key Words—6-Methoxybenzoxazolinone, hydroxamic acids, wheat, photoperiod, temperature.

INTRODUCTION

The timing of the onset of breeding of microtine rodents in natural populations can be highly variable. In field populations of *Microtus montanus*, the montane vole, the initiation of reproductive activity is correlated with the beginning of vegetative growth of their plant food resource in the spring, rather than at a

specific daylength (Negus et al., 1977). These herbivorous rodents eat primarily grasses and sedges, consuming fresh plant material when available during the growing season. Sanders et al. (1981) identified a factor derived from young winter wheat, 6-methoxybenzoxazolinone (6-MBOA), which increases uterine weight in female *M. montanus* and ovarian weight in *Mus musculus*. Using this compound in field studies, Berger et al. (1981) found that nonbreeding winter populations of *M. montanus* could be stimulated to breed several months before the onset of vegetative growth in the spring. Increases in reproductive function were observed in both sexes. In the laboratory, 6-MBOA increases litter size and frequency in this species (Berger et al., 1986a). This compound has been tested on other vertebrate species and has been found to have stimulatory effects on female reproductive function in rats (Butterstein et al., 1985), kangaroo rats (Rowsemitt, 1984), bobwhite quail (Berger et al., 1986b), rabbits, and mink (Berger and Negus, unpublished).

Prior to the work of Sanders et al. (1981), interest in 6-MBOA and related compounds centered on their role in the defense of young crop grasses against insect predators such as aphids (Argandona et al., 1980, 1981), corn borers (Klun and Brindley, 1966), and other pests. Although 6-MBOA is reported to be absent from intact plants, its precursor, 2,4-dihydroxy-7-methoxy-(2H)-1,4-benzoxazin-3(4H)-one (DIMBOA) has been reported in several species of the family Poaceae (Virtanen and Hietala, 1960; Loomis et al., 1957; Tang et al., 1975). This compound, present as a glucoside in intact tissue, is converted by enzymatic hydrolysis to the aglycone (DIMBOA) after tissue damage. The latter compound is unstable and decomposes to 6-MBOA (Figure 1) (see Virtanen and Hietala, 1960, for review). Therefore, 6-MBOA is formed when the plant's predator masticates the plant tissue. Argandona et al. (1981) examined the distribution of hydroxamic acids in wheat (*Triticum durum*) as an indicator of the major hydroxamic acid present, DIMBOA. On a per gram basis, maximum levels were reached in shoots by the fourth day after germination. Similar results were observed with *Triticum aestivum*, *Secale cereale*, and *Zea mays*. For *Triticum durum*, hydroxamic acid levels drop in old leaves, and new leaves on 30-day-old plants have lower levels than new leaves on younger plants. The authors did not report the environmental conditions under which the plants were grown.

Microtus montanus and some related species live in habitats with a high

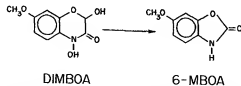


FIG. 1. Chemical structure of 6-MBOA and its precursor DIMBOA.

degree of temperature and rainfall variability on a year-to-year basis. The freshly sprouted grasses which they appear to use to cue the onset of reproduction (Negus et al., 1977; Berger et al., 1981) are growing under different temperature and photoperiod conditions in different years. While laboratory studies examining the distribution of derivable 6-MBOA have been performed (Argandona et al., 1981; Klun and Robinson, 1969), no attempts have been made to examine variations in 6-MBOA in young grasses growing under the range of conditions typical of natural habitats. We are currently involved in field studies of available 6-MBOA in wild grasses which are food resources for *M. montanus* and other microtine rodents. In order to more closely examine the effects of environmental variables on 6-MBOA availability in grasses, we have chosen to use winter wheat as a model system to examine the concentrations of derivable 6-MBOA in grasses grown under various photoperiod and temperature regimes.

METHODS AND MATERIALS

General. Winter wheat seeds were soaked in tap water for 1 hr, then planted 24 hr later in Vermiculite and grown in an environmental chamber with high-pressure sodium and metal halide high-intensity discharge lamps. Light intensity was 0.5 mmol photosynthetically active radiation/m²/sec. Four days after planting, triplicate 1.0-g samples of whole plants were extracted for derivable 6-MBOA. Samples were collected 3 hr after the onset of light. Entire shoots were utilized, cutting at the seed. Plant material was ground with sand to disrupt cell walls, incubated in distilled water for 1 hr at room temperature to allow enzymatic conversion to the aglycone, boiled for 30 min to allow conversion to 6-MBOA, then extracted three times with redistilled reagent-grade dichloromethane and dried under nitrogen. The dried samples redissolved in CH₃OH were analyzed for 6-MBOA by GC-MS. A Dupont model DP102 instrument equipped with an integrator and a SP2250 GC column isothermal at 200°C was used. The peak for the mass ion at *m/z* 165 was the most intense in the fragmentation spectrum and was integrated.

A standard curve was obtained using 0.06, 0.6, and 1.2 µg injections of a pure 6-MBOA in CH₃OH solution and was reproducible to ±5% at the low-weight end of the curve. Statistical comparisons were analyzed by linear regression unless otherwise specified.

Regimen I. 6-MBOA Concentrations under Various Photoperiods. Plants were grown under 8, 10, 14, and 16 hr of light per day with a temperature cycle of 12 hr at 25°C and 12 hr at 15°C such that the warm portion of the day occurred during the light and was symmetrical with the temperature regime.

Regimen II. 6-MBOA Concentrations under Various Temperatures. Plants were grown in 12 hr of light per day under the following temperature regimes

(°C): 35:15, 30:20, 20:10, 15:5, with the higher temperature given during the light portion of the day.

Regimen III. Age and Distribution Profile of 6-MBOA. Plants were grown under 12 hr of light per day with a daytime temperature of 30°C and a nighttime temperature of 20°C. Whole plant samples were taken at days 4, 9, and 16; 1.0-g samples of the first centimeter above the seed were also taken at days 4, 9, and 16. On day 9, the distribution of 6-MBOA within plants was examined by extracting 1.0-g samples of the first, fourth, and tenth centimeter above the seed.

Regimen IV. 6-MBOA Contents of Roots and Seeds. The 6-MBOA contents of roots and seeds were determined. Triplicate 1.0-g samples of roots were extracted from the 20°/10° sample in regimen II. Triplicate 1.0-g samples of unspouted seeds were ground to a powder and extracted.

RESULTS

Regimen I. Longer daylengths decrease the amount of 6-MBOA present per gram of plant material ($r^2 = 0.852$, $P < 0.001$) (Figure 2). There was no relationship between photoperiod and plant height ($r^2 = 0.077$; $P = 0.68$). Plant height ranged from 2.0 cm in 10 hr of light per day to 5.0 cm in 14 hr of light per day.

Regimen II. Increases in day and night temperatures decrease 6-MBOA content per gram of plant material ($r^2 = 0.867$, $P < 0.001$) (Figure 3). Plant height decreased with increasing temperature ($r^2 = 0.996$; $P = 0.039$). Height ranged from 7 cm in the 35°/25° regime to 0.5 cm in the 15°/5° regime.

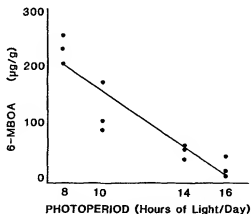


FIG. 2. Effect of photoperiod on derivable 6-MBOA in 4-day-old wheat plants. Regression: $y = -25.82x + 425.31$. $r^2 = 0.852$, $P < 0.001$.

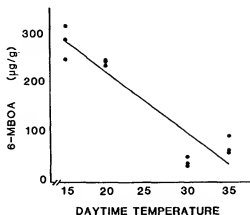


FIG. 3. Effect of temperature on derivable 6-MBOA in 4-day-old wheat plants. (Night-time temperatures are 10° lower.) Regression: $y = -13.52x + 506.78$, $r^2 = 0.867$, $P < 0.001$.

Regimen III. Age has a highly significant effect on derivable 6-MBOA, with highest levels found in the 4-day sample ($r^2 = 0.650$, $P = 0.0156$) (Figure 4A). Comparisons of only the first centimeter above the seed yielded similar results with the 4-day sample having the highest concentration of derivable 6-MBOA ($r^2 = 0.944$, $P < 0.001$) (Figure 4B). Comparing the concentration of 6-MBOA in the first centimeter with that in the entire plant shows no significant difference at day 4, but significantly higher concentration in the first centimeter than in the whole plant at days 9 and 16 ($P = 0.001$, $P = 0.039$, respectively, Student's t test). This conclusion is supported by the results in Figure 5, showing concentrations in several portions of the plant ($r^2 = 0.976$, $P < 0.001$).

Figure 5 shows the distribution of derivable 6-MBOA within plants at 9 days of age grown under the conditions described above. The highest concentrations are found at the meristematic region at the base of the grass with decreasing concentrations in the higher portions of the plant.

Regimen IV. Root samples from 9-day-old plants as described above averaged $101.3 \mu\text{g}$ derivable 6-MBOA/g plant material (± 14.0 SD). Unsprouted seeds contained $3.3 \mu\text{g}$ 6-MBOA/g (± 5.8 SD).

DISCUSSION

To understand the availability of 6-MBOA in natural food resources of microtine rodents such as *Microtus montanus*, we have used one grass (wheat) as a model system. Under controlled environmental conditions in the laboratory, we have studied the effects of altering both photoperiod and temperature

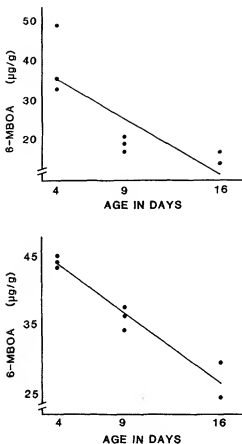


FIG. 4. Effect of age on derivable 6-MBOA in wheat. (A) Whole plant. Regression: $y = -2.05x + 44.78$, $r^2 = 0.650$, $P = 0.016$. (B) First centimeter. Regression: $y = -1.36x + 50.23$, $r^2 = 0.044$, $P < 0.001$.

on derivable 6-MBOA. While we recognize that the absolute values determined here for wheat are not likely to be equivalent to those found in the natural grasses which are food resources for microtine rodents, our purpose is to elucidate how the plants' investment in the production of the precursor to 6-MBOA varies under different growing conditions. Other work in progress examines 6-MBOA content of known food resources of *M. montanus* (Negus, Berger, and Epstein, in preparation).

Our results demonstrate that both photoperiod and temperature affect the concentration of derivable 6-MBOA in 4-day-old plants. Increasing the hours of light per day decreases the concentration of derivable 6-MBOA per gram of

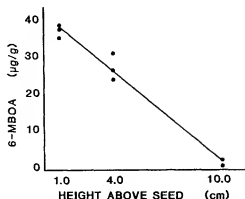


FIG. 5. Distribution of derivable 6-MBOA in the first, fourth, and tenth centimeter above the seed. Regression: $y = -3.73x + 41.86$. $r^2 = 0.976$, $P < 0.001$.

fresh plant material. Increasing the temperature also decreases the amount of derivable 6-MBOA. Since higher temperatures produce more biomass per plant, the temperature effect may be due, in part, to the fact that more individual plants are required to make a gram of material in the wheat grown at lower temperatures than at the higher temperatures. The result is that more meristematic material is present in the samples at lower temperatures. Since 6-MBOA occurs in higher concentration in the first centimeter, a 1.0-g sample of shorter plants will contain more individual plants and thus more first-centimeter portions (the meristematic region in grasses) than a 1.0-g sample of taller plants and will therefore contain more 6-MBOA if plant height is the important factor. The photoperiod effect on 6-MBOA concentrations cannot be explained in this manner because the plant height did not vary in a systematic fashion with photoperiod. Our data demonstrate that, regardless of photoperiod and temperature conditions, freshly sprouted wheat seedlings contain significant amounts of derivable 6-MBOA, with approximately a fivefold range in 6-MBOA on a per gram fresh weight basis under the different treatments.

The results of our examination of the distribution of 6-MBOA within the plant are consistent with those of other workers. Highest concentrations were found at the first centimeter above the seed, with decreasing concentration at greater heights. This is in agreement with the results of Klun and Robinson (1969) for several strains of corn and with those of Argandona et al. (1981) for wheat. Direct comparisons of our results with those of other workers are of limited value for several reasons. The intraspecific differences in 6-MBOA can be very dramatic in inbred strains of crop grasses. Examination of 11 strains of corn in one study revealed almost a 10-fold range in 6-MBOA concentrations (Klun and Brindley, 1966). Furthermore, the growing conditions varied substantially between studies. In spite of high variability in actual 6-MBOA content

in various grasses, interspecific generalities are emerging regarding the distribution of 6-MBOA in those grasses which contain it.

Our age profile, with samples taken at days 4, 9, and 16, showed decreasing levels from day 4, whether the entire grass was sampled or the first centimeter only was used. Other age profile work shows similar results. Argandona et al. (1981) show decreases in hydroxamic acids in wheat after four days of age. Argandona et al. (1980) took their first sample at 10 days of age. For wheat, decreases occurred after day 10; for rye (*Secale cereale*) days 10 and 16 produced similar levels of hydroxamic acids with decreases starting at day 22. Klun and Robinson (1969) reported 6-MBOA concentrations based on plant height in several strains of corn (*Zea mays*). Their first sample, at 6 in., was the highest value for every strain examined. Therefore, highest concentrations are found in the young plants.

The relevance of the root levels of 6-MBOA to microtine food resources is not clear. Behavioral studies of *Microtus breweri* have shown a seasonal shift in preference for various portions of their major food resource, the beach grass, *Ammophila brevifoligulata*. In late summer, the roots are the preferred portion of the plant (Rothstein and Tamarin, 1977). However, our own and other studies of 6-MBOA in roots have been from young actively growing plants; we know nothing about the availability of 6-MBOA in a perennial grass late in the growing season. Derivable 6-MBOA has been found in the roots of wheat, rye, and Job's tears (Tang et al., 1975). The availability of 6-MBOA in roots may be of importance to reproductive patterns of root-eating subterranean rodents such as pocket gophers (Geomysidae).

Our inability to detect significant amounts of derivable 6-MBOA in wheat seeds is consistent with the results of Argandona et al. (1981). They found no hydroxamic acids in wheat seeds (*Triticum durum*). This fact is of particular interest regarding the breeding patterns of some heteromyid rodents. Certain desert-adapted kangaroo rats, primarily seed-eaters, include fresh vegetation in their diet when it is available after rainfall (Reynolds, 1960; Reichman and Van De Graaff, 1975). Breeding correlates with the availability of the fresh vegetation. In one species of kangaroo rat, *Dipodomys ordii*, 6-MBOA causes uterine hypertrophy (Rowsemitt, 1984). If 6-MBOA is absent from the kangaroo rats' usual diet but is present in the fresh vegetation ingested after rainfall, it may provide a cue for breeding in these animals.

The exact results of this work are not expected to be quantitatively comparable to the values found in field populations of various grasses. The light intensity used was lower than full sunlight. Also the 6-MBOA content of different species of crop grasses, as well as our own unpublished work, show that interspecific differences are substantial. Argandona et al. (1980) demonstrated that hydroxamic acids are present in wheat and rye, but nondetectable in barley. Therefore, not all grasses contain these compounds. The main purpose here has been to examine the range of phenotypic plasticity of this parameter in one

system. The results presented here suggest that 6-MBOA concentrations may be higher per gram of plant tissue in grasses sprouting in the colder temperatures and shorter photoperiods of early spring than in those sprouting later in the season when temperatures are warmer and photoperiods are longer. Therefore, the animals may encounter higher concentrations of 6-MBOA in newly sprouted grass at the beginning of the breeding season than in grasses sprouting later. The dynamic aspects of 6-MBOA concentrations in plants may prove to be an important factor in breeding patterns of many herbivores.

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